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Enzymatic efficiency of the decomposing microbiota: what does really matter for aquatic macrophytes invasions?

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ABSTRACT

Biological invasions have negative impacts on different ecosystem-level functions, such as nutrient cycling. In aquatic environments, exotic litter can change the activity of the decomposer microbiota. We tested whether litter quality, litter decay, and enzyme activity differed between native *Egeria densa* and exotic *Hydrilla verticillata*. The invasive plant presented higher lignin and lower cellulose content than the native plant. Both species showed rapid fibre decay in the first five days. *E. densa* had higher cellulose and hemicellulose decay than *H. verticillatta*. Although the species did not exhibit differences in enzyme activity over time, *E. densa* had a higher enzymatic efficiency than *H. verticillata*. This differential enzymatic performance can cause changes in the mineralisation processes of the invaded environments. The lower decomposition rates for invasive litter, associated with differences in litter quality, could increase the amount of particulate organic material in invaded environments.

Keywords: environmental impact, freshwater, litter decay, microbiological processes, refractory litter

Introduction

The aquatic plant community plays a significant role in structuring aquatic environments, nutrient cycles, and biota maintenance (Wetzel 2001). These plants spread, grow, and reproduce rapidly, even under suboptimal conditions (*i.e.*, under intense competition), presenting rapid metabolism and large biomass in eutrophic ecosystems (Pieterse & Murphy 1990). Aggressive invaders can threaten biodiversity and ecosystem processes (Murphy *et al.* 2019). The submerged *Egeria densa*, along with *Hydrilla verticillata*, are two of the main noxious aquatic weeds in reservoirs worldwide (Strange *et al.* 2019), and (Purcell *et al.* 2019). These plants present similar ecological strategies and growth forms that potentially occur in similar habitats and have a wide ecological range (Mony *et al.* 2007). The genera *Egeria* and *Hydrilla* (Hydrocharitaceae) have been a focus of concern in reservoirs worldwide because of changes in water flow, displacement of native vegetation, and negative impacts on non-plant species (True-Meadows *et al.* 2016). Their large biomass (Bianchini Jr. *et al.* 2010; Silveira & Thomaz 2019) increases the amount of litter and modifies habitat conditions (Dainez-Filho *et al.* 2019).

Macrophyte biomass is preferably degraded by invertebrates and associated microbiota (Bornette & Puijalon 2011). The participation of shredder invertebrates in decomposition in tropical and subtropical aquatic systems has been questioned, highlighting the

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involvement of fungi and bacteria in the degradation of organic matter (Albertoni *et al.* 2020). Moreover, a large portion of the macrophyte biomass, available in the form of litter, participates in microbial assemblage metabolism. In aquatic environments, enzymatic activity of microbial communities is responsible for degrading dissolved organic carbon (DOC) and particulate organic carbon (POC). POC comprises lignocellulosic fibres such as cellulose, hemicellulose, and lignin. These fibres are the refractory litter fraction, mainly mineralized by microbial hydrolytic enzymes (Bottino *et al.* 2019). Aquatic decomposition occurs through aerobic and anaerobic processes. In the litter of submerged plants, aerobic decomposition acts primarily on DOC, mainly resulting in carbon dioxide, water, and humic compounds (Bianchini Jr. 2003). POC, on the other hand, is mostly anaerobically decomposed once anaerobiosis is reached in the first centimetres of the sediment (Nedwell 1984). Carbon dioxide, methane, humic substances, mercaptans, molecular hydrogen, and sulfide hydrogen are generated by anaerobic degradation (Sanderman & Amundson 2008).

Altered litter quality can inflict profound changes in the structure and function of the decomposer community. These changes influence the abundance, identity, and activity rates of sediment biota (Wolf & Klironomos 2005). According to Kourtev *et al.* (2002), changes in the composition of plant community species can result in changes in the enzymatic activities of microbiota, mainly in heavily invaded communities. Several studies corroborate changes in the microbiota and consequent changes in nutrient cycling in invaded areas (Kourtev *et al.* 2003; Mincheva *et al.* 2014; Stefanowicz *et al.* 2016). Consequently, nutrient cycling at the ecosystem scale may be slowed down by exotic invasions (Godoy *et al.* 2010). However, the relationship between the quality of the invasive litter, microbial community composition and activity, and litter decomposition rates under invasion is still controversial (Cleveland *et al.* 2014). There is evidence that the microbial community can adapt to different litter qualities (Hoyos-Santillan *et al.* 2018). In addition, many plant invaders produce litter with high nutritional quality, further accelerating the nutrient cycling processes in invaded ecosystems (Arthur *et al.* 2012; Jo *et al.* 2017). Therefore, the study of invasive macrophyte decomposition is essential to understand the changes in the aquatic nutrient dynamics caused by the invasion in each context (Ehrenfeld *et al.* 2010).

Our study compared litter quality, litter decay, and anaerobic enzymatic decomposition of native *E. densa* and exotic *H. verticillata*. We hypothesised that fibre content and fibre decay differ among macrophytes with similar niches. Consequently, we believe that this invasive macrophyte promotes changes in the enzyme activity of decomposer microbes (Kourtev *et al.* 2003).

Materials and methods

Study area

The main rivers of the Parana basin are Paraná and Paraguay, which occupy most of the central southern South America (18 to 34° S and 45 to 68° W (Agostinho *et al.* 2007). The Porto Primavera hydroelectric reservoir (UHE Engenheiro Sergio Motta) is located on the Parana River between 53 and 52° W and 22 to 22° S. The reservoir area is 2,040 km^2, with a water volume of 15.7 billion m^3, and a total length of the longitudinal axis of 250 km (Bianchini Jr. *et al.* 2010). According to Sousa (2011), the first occurrence of *Hydrilla verticillata* (L.f.) Royle in the Parana River downstream from this reservoir dates back to 2005, and since then, this species has been found among the submersed macrophytes in the Paraná Basin. Reservoir Engenheiro Souza Dias (Jupiá hydroelectric), the first reservoir upstream of Porto Primavera, has a flooded area of 330 km^2, is 5,495 m long, and receives water from the Paraná, Tietê, and Sucuriu Rivers. There are various problems with infestations of submerged aquatic plants, such as native *Egeria densa* Planch., *Egeria najas* Planch. (Hydrocharitaceae), and *Ceratophyllum demersum* L. (Ceratophyllaceae), along with the occurrence of dams in the Tiete River and in areas of the Paraná River, which has higher water transparency (Velini *et al.* 2005).

Experimental design and sampling procedure

We collected fragments of *E. densa* and *H. verticillata* along the Jupiá and Porto Primavera reservoirs. At the same sites, water was collected using a Van Dorn bottle. The macrophyte samples were washed and dried in an incubator at 40 °C until a constant weight was achieved. We mixed water samples from both reservoirs and filtered them in a cellulose ester filter (Φ = 0.45 µm). Anaerobic incubations were carried out according to Bianchini Jr. *et al.* (2002). Anaerobic incubations represent a probable environment for decomposing the lignocellulosic fraction of submerged plant litter (Nedwell 1984; Bianchini Jr. 2003). We incubated the dried fragments of these macrophytes in 60 glass bottles (500 mL) at a 10 g dry weight (DW) ratio per L^{-1} of reservoir filtered water. Samples were kept in anaerobic conditions at 25 ± 1 °C in an incubation chamber. On sampling days (1, 3, 5, 10, 15, 20, 30, 40, 50, and 65), we collected the particulate organic matter (POM) from three incubations for each species. The POM mass was determined gravimetrically and converted into carbon-based POC, according to Wetzel (2001). We determined the lignin, hemicellulose, and cellulose contents by digestion of POM macrophyte samples followed by gravimetric analysis according to Allen *et al.* (1974), Han & Rowell (1996), and Clampton & Maynard (1938), respectively. We used one sub-sample of each incubation (3.0 g DW) for enzyme assays. The sub-samples were blended (Ultra-Turrax model T10; Germany) with 10 mL of acetate

buffer (50 mM, pH 5.2), sonicated (ultrasound Unique, Brazil), and centrifuged (3,000 × g, 30 min, 4 °C; Heraeus 122 Instruments, Megafuge 3.0R, Germany). We used the enzymatic extracts from the supernatant fraction, in the cellulase, xylanase, and peroxidase activity determination by spectrophotometric methods (Amersham Biosciences, Ultrospec 2100 pro, Sweden). We evaluated the cellulolytic activity C1 (endocellulases synergistic action of enzymes: EC 3.2.1.4 and exocellulases: EC 3.2.1.91) using the method proposed by Mandels *et al.* (1976). We determined xylanase activity (EC 3.2.1.8) using the method proposed by Highley (1997). We quantified the reducing sugars released by the enzyme action on a specific substrate (pure cellulose filter Whatman n°1 for cellulase and xylan for xylanase) using the Somogy method (540 nm) (Somogyi 1952). The enzymatic activity of peroxidase was determined using a modified version of Frew *et al.* (1983). We monitored the absorbance at 510 nm for 180 s after the addition of the enzyme extract. We defined one unit (μmol.min-1 mL-1 g-1) as the amount of enzyme that catalyses the conversion of one micromole of substrate per minute under the assay method's specified conditions.

Data analysis

We organised the fibre decay (FD) data as a function of the enzymatic activity (EA) to determine the enzymatic efficiency of each enzyme in each litter (FD/EA). We used a MANOVA and a *post hoc* ANOVA to test for significant differences in fibre content between the two species. We used the generalised linear mixed model with temporal pseudoreplication to test for significant differences in litter decomposition for these two species. These models included experimental time as a categorical factor (each resampling date as one level) and all possible interactions between pairs of these factors. We assumed a Gaussian distribution for the effects on the accumulated fibre decay data, enzymatic activity, and enzymatic efficiency data. All models were implemented in the R package lme4 (function 'lme') and all statistical analyses were performed in the R statistical environment (R Development Core Team 2020).

Results

The Figure 1 shows the proportions of the hemicellulose, cellulose, and lignin constituents of *E. densa* and *H. verticillate* (Fig. 1A). The fibre content of *E. densa* was significantly different from that of *H. verticillata* (MANOVA; approx. F= 25.736; p= 0.037). In both species, the main constituent fibre was hemicellulose (49.4 % in *E. densa* and 50.3 % in *H. verticillata*), and there were no significant differences in hemicellulose percentage between these plants (ANOVA; F= 0.702; p= 0.449). The percentage of cellulose in *E. densa* (30.6 %) was higher than that in *H. verticillata* (21.5 %) (ANOVA; F= 18.376; p= 0.013). The percentage of lignin in *E. densa* (20.05 %) was lower than that in *H. verticillata* (28.2 %) (ANOVA; F= 14.039; p= 0.020). Similar patterns of fibre decay were observed in both species (Fig. 1B, C). The first five sampling days presented a quick fibre decay for all fibres. Over the remaining days, the fibre decay was slow.

The Table 1 shows the GLMM results comparing accumulated fibre decay, enzyme activity, and accumulated efficiency between both species. Figure 1C shows that higher hemicellulose (GLMM; p= 0.006) and cellulose (GLMM; p= 0.023) accumulated fibre decay in *E. densa* than *in H. verticillata*. As for lignin, the data did not present differences between *E. densa* and *H. verticillata* (GLMM; p= 0.115).

Table 1. Results of the Generalized Linear Mixed Models adjusted to accumulated fibres decay, enzymes activities and enzymes accumulated efficiencies data between *Egeria densa* and *Hydrilla verticillata* anaerobic litter decomposition.

Measure	Variable	Value	Std.Error	DF	t-value	p-value
Accumulated fiber decay	Intercept	30.379	1.217	18	24.951	
	Hemicelluloses	-5.339	1.722	18	-3.101	0.006
	Intercept	26.612	1.237	18	21.515	
	Cellulosis	-4.329	1.749	18	-2.475	0.023
	Intercept	33.749	1.868	18	18.068	
	Lignin	-4.372	2.642	18	-1.655	0.115
Enzyme activity	Intercept	0.001	0.001	18	4.540	
	Xylanase	0.001	0.001	18	1.350	0.194
	Intercept	0.000	0.000	18	7.900	
	Cellulase	0.000	0.000	18	1.269	0.220
	Intercept	13.030	1.920	18	6.786	
	Peroxydase	-2.061	2.715	18	-0.759	0.457
Enzyme efficiency	Intercept	25175.06	1276.905	18	19.716	
	Xylanase	-12342.62	1805.817	18	-6.835	<0.001
	Intercept	149800	6695.594	18	22.372	
	Cellulase	-74820	9469.000	18	-7.902	<0.001
	Intercept	2.014	0.0624	18	32.280	
	Peroxydase	-0.386	0.088	18	-4.372	<0.001

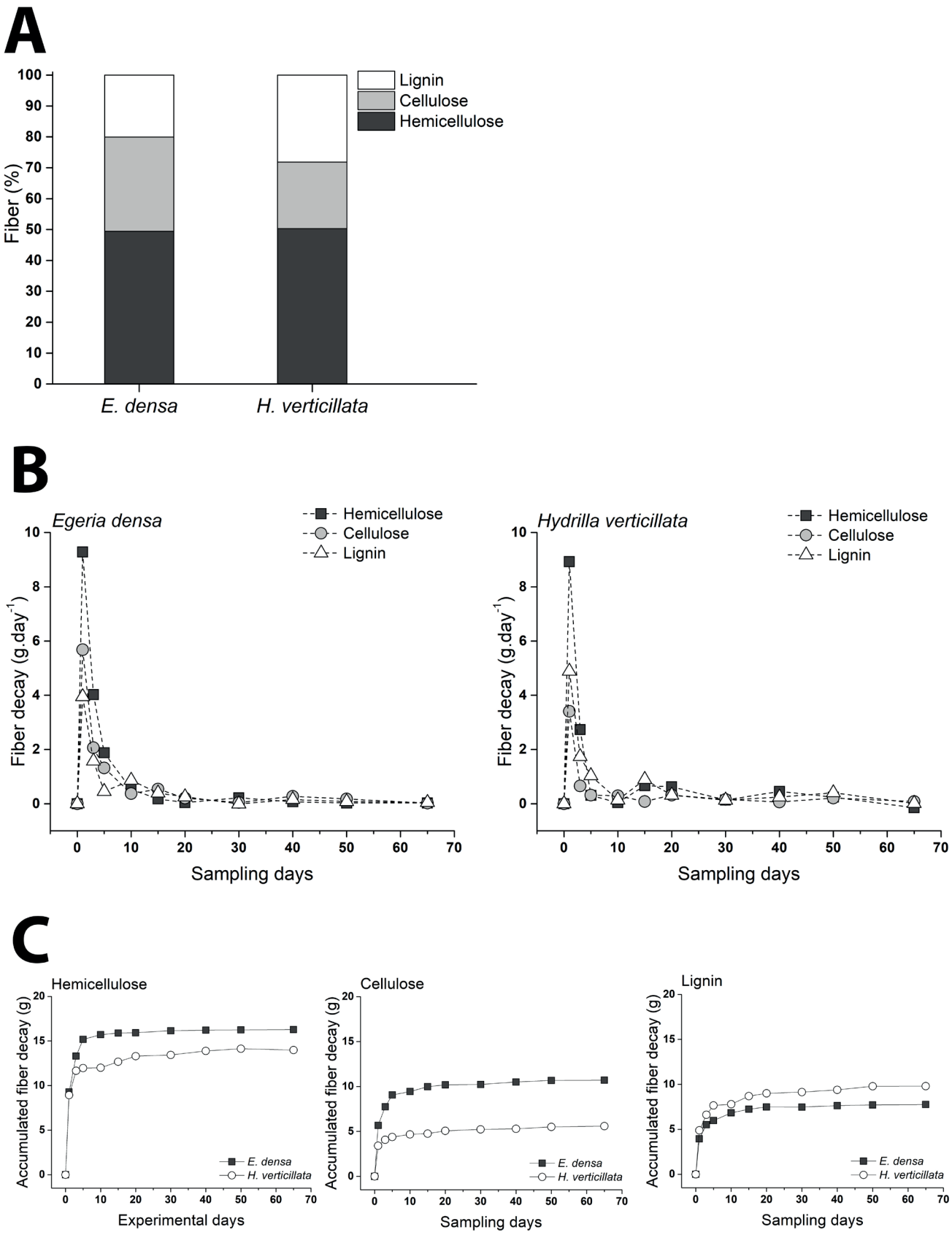

Figure 1. *Egeria densa* and *Hydrilla verticillata* hemicellulose, cellulose, and lignin content and standard deviation bars (**A**), litter fibre decay (**B**), and accumulated litter fibre decay (**C**).

The Figure 2 shows the enzymatic activities of *E. densa* and *H. verticillate* (Fig. 2A). There were no significant differences in cellulase (GLMM; p= 0.194), xylanase (GLMM; p= 0.220), and peroxidase activity (GLMM; p= 0.457) between the species. The highest peroxidase activity was observed on the 40th day for *E. densa* and on the 20th day for *H. verticillata*. The cellulase activity increased at the beginning and remained similar over time. For both species, xylanase activity increased at the beginning and on the 20th day. We observed higher enzymatic efficiency in *E. densa* when compared with *H. verticillata* for xylanase (GLMM; p< 0.001), cellulase (GLMM; p< 0.001) and peroxidase (GLMM; p< 0.001).

Discussion

Egeria densa presented higher cellulose and hemicellulose decay than *Hydrilla verticillatta*. The enzymatic activity in *E. densa* litter was more efficient for both fibres than *H. verticillata*, even with similar hemicellulose content. These differences may have occurred due to the higher concentration of lignin in *H. verticillata,* which may have inhibited access to hemicellulose. In general, hydrolysis of lignocellulose is a limiting step during anaerobic digestion of plant litter since recalcitrant lignin protects cellulose and hemicellulose against microbial/enzymatic attack by coating them (Taherzadeh & Karimi 2008). However, despite the higher lignin content in *H. verticillata*, our results showed no differences between lignin decay in these plants. In anaerobic environments, lignin presents limited degradation because the enzymatic apparatus for processing this fibre generally uses oxygen as an electron acceptor (Tuomela *et al.* 2000). Furthermore, *E. densa* lignin appears to be naturally recalcitrant. In comparison to other species, as described in the study by Koyama *et al.* (2014), regardless of lignin concentration, *E. densa* showed a lower delignification rate and higher alkali consumption during the alkaline delignification process.

The highest xylanase production compared to cellulase was associated with molecular structural factors. Hemicellulose polymers are more readily hydrolyzable than cellulose (Pérez *et al.* 2002). Despite the greater complexity of hemicellulose, with short side chains of different sugars (*e.g.*, D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, acid 4-O-methyl-glucourônico, D-galactourônico, and D-glucourônico) (Pérez *et al.* 2002), which entails the need for a greater diversity of hydrolysing enzymes, which presents binding sites that are more accessible than cellulose (Gilbert & Hazlewood 1993) without forming aggregates, even when co-crystallized with cellulose chains (Cunha-Santino & Bianchini Jr. 2008). Sciessere *et al.* (2011) and Nunes *et al.* (2011) considered the higher xylanase activity observed in litters of *Salvinia* sp., *Eichhornia azurea*, *Cyperus giganteus, Ricciocarpus natans, Oxycaryum cubense,* and *Cabomba furcata* because of the easy access of this fibre for the microorganisms when compared with cellulose access.

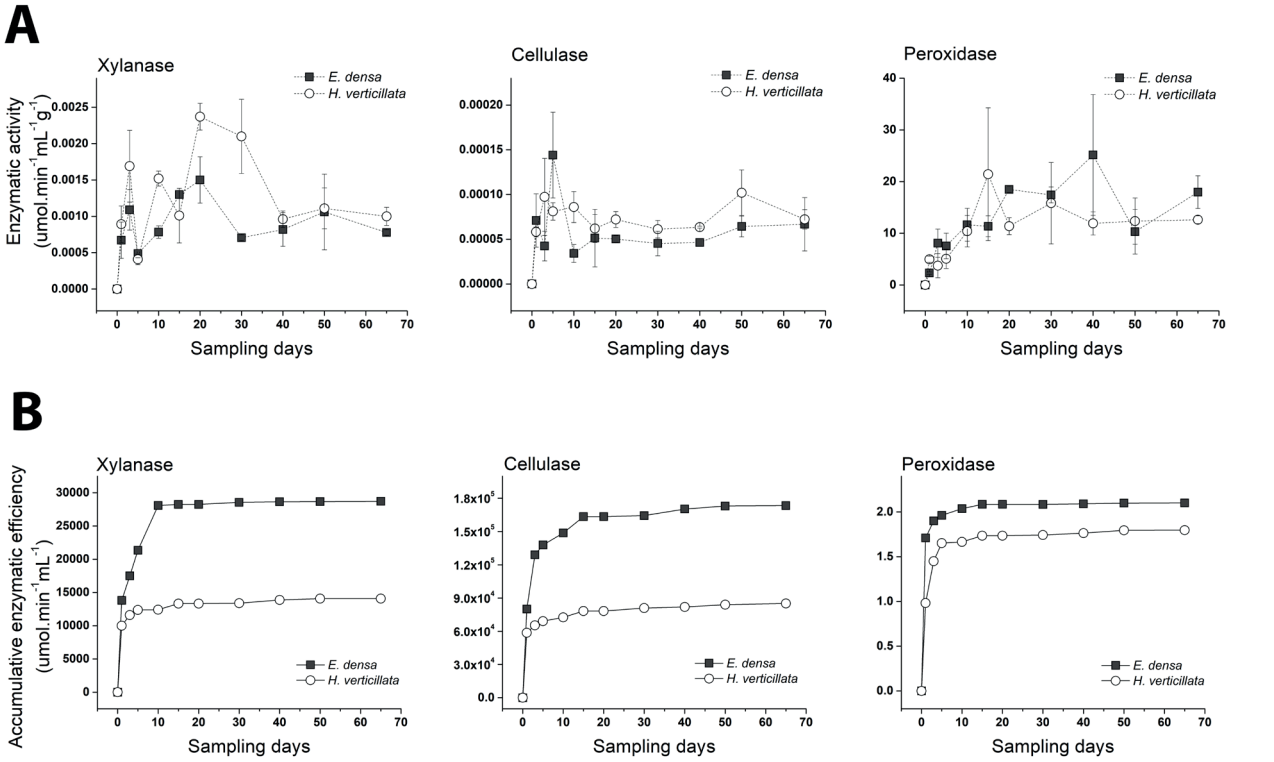

Figure 2. Comparison of enzymatic activity (**A**) and accumulative enzymatic efficiency (**B**) between *Egeria densa* and *Hydrilla verticillata* during anaerobic decomposition.

The activity of oxidative enzymes (*e.g.*, peroxidases) is related to refractory substrate materials, such as lignin. The most recalcitrant quality of lignin, compared with cellulose and hemicellulose, requires a high proportion of energy allocated to this compound (Kourtev *et al.* 2002; Margida *et al.* 2020). With a more refractory substrate, there is a need for increased enzyme activity, which decreases enzymatic efficiency (Fioretto *et al.* 2000). Another factor influencing the high peroxidase activity is its general nature. Sources other than lignin fibres can induce peroxidase production. A vast number of processes, based on C-C bond cleavage, such as phenolic compounds, polysaccharides, cellobiose (Sinsabaugh *et al.* 2009), depolymerisation of lignin phenols and methoxylated benzoic replacing compounds, hydroxylation and oxidation, and aromatic ring cleavage, use H_2O_2 as an acceptor (Lynch & Hobbie 1989; Sun *et al.* 2018). Refractory compounds (*e.g.*, lignin) are mineralized at low speeds (Fioretto *et al.* 2000; Kourtev *et al.* 2002). However, the pattern decay of lignin in *E. densa* and *H. verticillata* was the same as that of the other fibres. Thus, the high proportion of lignin in these plants and the presence of other compounds that induce peroxidase can cause high and rapid enzyme productivity, even under anaerobic conditions. Quantitatively, the decomposition of lignin is quite significant, as the survey conducted by Cunha-Santino & Bianchini Jr. (2008) showed that the percentage of lignin in the supporting tissues of macrophytes can reach 33.4%, which is an important source of autochthonous carbon.

The decomposer microbiota associated with *E. densa* litter presented significantly higher enzymatic activity efficiency than that associated with *H. verticillata*. The release of extracellular enzymes is species-dependent and is influenced by temperature, moisture, pH, and the quality and quantity of available substrate (Sinsabaugh & Linkins 1990; Fioretto *et al.* 2000). The quality of the substrate affects decomposition mostly by controlling microbial colonisation, growth, and activity (Sinsabaugh *et al.* 2009). The composition of the substances found in different species may significantly change the metabolic composition of the decomposer microbiota (Harner *et al.* 2009). Kourtev *et al.* (2002) found significant differences among individual enzyme activities in four litter types of different species in the soil of plants, and the enzyme activity of the two native species was different from that of the two exotic species considered. Our results indicate the same enzyme activity for both species, despite the differences found in their fibre contents. In addition, Koyama *et al.* (2014) showed that the anaerobic digestibility of submerged macrophytes is regulated by the lignin content, explaining our results.

The differences found for the enzymatic decomposition between *E. densa* and *H. verticillata* are qualitative, related to how enzymes operate in the litter. This is because the lignocellulose index (LCI = lignin/[lignin + hollocellulose]) controls the microbial allocation of extracellular enzymes (Margida *et al.* 2020). This differential enzymatic performance changes environmental mineralisation processes. Our results suggest a more substantial mineralisation time for *H. verticillata* than for *E. densa*. The most recalcitrant litter of this invasive plant can increase the retention time of POM in the invaded environment, resulting in high levels of turbidity (Wetzel 2001), sedimentation processes (Bates *et al.* 1984), and an increase in humic substances in the sediment (Cunha-Santino & Bianchini Jr. 2008).

Hydrilla verticillata is considered one of the worst aquatic weeds globally (Hershner & Havens 2008). Our results suggest that the introduction of this invasive macrophyte in neotropical reservoirs could promote changes in litter quality and decomposition processes. Consequently, there can be changes in the ecosystem in the medium and long term as an accumulation of particulate refractory material in the sediments and an increase in anaerobic heterotrophy. However, field studies are needed to confirm the effects of this invader on nutrient cycling in invaded environments.

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